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13. ABSTRACT (Maximum 200 words) Our work has effectively merged <u>in situ</u> measurements of specific microbial metabolisms using radio-labeled growth substrates with 16S rDNA phylogenetic characterization of the microbial community in the same sample. Our group worked with pure cultures of bacteria and geochemically well-characterized systems and made numerous rate measurements with radio-labeled substrates. We conversed on a successful molecular method to quantify the incorporation of radio-labelled compounds into DNA by specific groups of bacteria.		
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FINAL REPORT

GRANT #: N00014-00-1-0751

PRINCIPLE INVESTIGATOR: Allan H. Devol

INSTITUTION: University of Washington, School of Oceanography

GRANT TITLE: Tracing Substrate Utilization by Specific Marine Sedimentary Microorganisms Using DNA Hybridization-Capture Technology

AWARD PERIOD: 1 June 2000 - 31 May 2001

OBJECTIVE: Our primary objective was to develop a molecular technique to trace the *in situ* uptake of specific microbial growth substrates by specific microorganisms in marine sediments. We also sought to more thoroughly understand the cycling of methane and sulfate in anoxic muds.

APPROACH: Our approach was to merge *in situ* rate measurements of specific microbial metabolisms using radio-labeled growth substrates with 16S rDNA phylogenetic characterization of the microbial community in the same sample. To do this required the development of a new molecular method. Our group worked with pure cultures of bacteria and geochemically well-characterized systems during this period of technical development. Numerous rate measurements were made in marine waters (thymidine uptake, uridine uptake) and anoxic marine muds (anoxic acetate respiration, acetoclastic methanogenesis, anaerobic methane oxidation) and radio-labeled DNA was extracted from these experiments. Using these DNA extracts, we have converged on a successful molecular technique to quantify the incorporation of radio-labeled compounds into DNA by specific, phylogenetically-defined groups of prokaryotes. With this information, we are able to determine the relative metabolic rates of specific groups of interest.

ACCOMPLISHMENTS: We completed our primary objective: the development of a method to analyze the radioactivity of DNA from a specific group of prokaryotes for the purpose of tracing radio-labeled substrates. Critical to the overall development of the method were two key technical breakthroughs: 1) the use of DNA polymerase to label targeted DNA with biotin for subsequent affinity capture on streptavidin-coated beads, and 2) synthesis of ³²P-labelled, size-matched, internal standards that are used to quantify the recovery of targeted DNA. With this technique in hand, we went on several research cruises in Puget Sound to measure the incorporation of ³H-methyl thymidine into DNA. We also constructed 16S rDNA clone libraries. Additionally, we sampled Saanich Inlet, an anoxic fjord on Vancouver Island, and conducted measurements of methane cycling in the anoxic sediments at this location. Porewater profiles of methane and sulfate were determined and sediments from various depths below the sediment water interface were incubated with 1-¹⁴C acetate and 2-¹⁴C acetate.

Specifically, our work toward tracing the utilization of specific organic molecules by a specific subset of the total microbial community involved the development of a quantitative method to separate DNA from a specific group of organisms from a complex mixture of ³H- or ¹⁴C-labelled DNA so that the radioactivity of DNA from specific organisms could be measured. At the initial stages, we were focused primarily on hybridizing DNA to biotin-labeled oligonucleotide probes and capturing the hybrids by affinity binding on streptavidin-coated beads, but our recoveries were quite low. Subsequently, we discovered that DNA polymerase, along with specific primers and biotin-labeled nucleotides, could be used to label the targeted gene (16S) multiple times, and radically increase recovery of the targeted DNA on the streptavidin-coated beads. We have termed this new molecular development, "specific nucleic acid polymerase biotinylation and affinity capture" or "SNAP-BAC". In addition, we now know that this method is quantitative; we determine the yield of ³H or ¹⁴C in DNA by normalizing to the recovery ³³P-labelled DNA standards added at the beginning of the procedure.

As a "test case" for tracing specific substrates to specific DNA, we studied the incorporation of ³H-methyl thymidine into DNA. The ³H-methyl thymidine method is a classic measure of bacterial growth in marine systems, and was a convenient biochemical pathway to test our method since we were assured that the DNA would be sufficiently radio-labeled. 16S rDNA clone libraries constructed using bacterial primers revealed that three subclasses of the Proteobacteria and the Cytophaga-Flavobacterium (C.-F.) cluster were represented in the water column of Puget Sound. The alpha-proteobacteria and C.-F. cluster were particularly prevalent and we targeted this group in our experiments. We collected water samples in Puget Sound, and incubated with ³H-methyl thymidine. Then using primers specific for alpha-Proteobacteria and the C.-F. cluster we applied the SNAP-BAC technique and determined that these groups were responsible for 38 ± 6% and 56 ± 15% of the total ³H-thymidine incorporation. To the best of our knowledge, this is the first culture-independent, *in situ*, rate measurement of DNA synthesis by a specific microorganism in a mixed microbial community.

Our work with the cycling of acetate in anoxic muds has helped us understand the biochemical efficiency of DNA synthesis with respect to substrate respiration. This is critical information since future experiments must be designed such that sufficient radio-label (¹⁴C, ³H, ³³P, etc.) from the growth substrate or pollutant will ultimately be incorporated into DNA. Acetate is an ideal molecule for this type of experiment since it enters the citric acid cycle directly where carbon (and, hence ¹⁴C) for the synthesis of purines and pyrimidines (from a-ketoglutarate and oxaloacetate) and ribose 5-phosphate (from oxaloacetate) is ultimately synthesized into DNA. Further, carbon from hydrocarbons also arrives at the citric acid cycle in the form of acetate following transformation into fatty acids and subsequent beta-oxidation.

In situ rates of acetate oxidation and acetoclastic methanogenesis were determined by measuring the ^{14}C activity of CO_2 and CH_4 respectively in Saanich Inlet muds. Anaerobic methane oxidation rates may be indirectly determined from these data also. In addition, we conducted complementary experiments to trace the incorporation of ^{14}C from acetate into DNA. The ^{14}C activity of the DNA showed that the ratio of acetate oxidation to acetate incorporation into DNA was approximately 100:1; this ratio varied by factor of two between the 1- ^{14}C acetate and 2- ^{14}C acetate experiments with the 2- ^{14}C acetate being consistently higher.

SIGNIFICANCE: Our approach may ultimately be applied to study the cycling of a variety organic molecules in all types of natural environments including anoxic marine sediments. The biogeochemical role of specific organisms within the microbial community may be investigated *in situ* by isolating specific DNA. This could have an important impact on our species-level understanding of the molecular systematics of organic matter cycling and degradation; only through study at this level will critical consortial relationships truly be known *in situ*. For example, our technique could be used to identify what group of bacteria is responsible for the *in situ* bioremediation of sediments contaminated with organic molecules such as petroleum hydrocarbons. Once the general phylogeny of the contaminant-degrading organism is known (i.e. delta-proteobacteria) subsequent sets of experiments could be designed to ameliorate growth conditions for the target organism and ultimately arrive at a treatment solution for the site. While these experiments are not a trivial undertaking in the laboratory this approach could ultimately provide significant cost savings. Further, we anticipate that this technique will yield substantial information in the study of global biogeochemical cycles.

PATENT INFORMATION: We have contacted our patent office at the university of Washington and submitted our disclosure. We are first waiting the acceptance of our manuscript before proceeding.

PUBLICATIONS AND ABSTRACTS:

Van Mooy, B.A.S., Devol, A.H., and Keil, R.G. (submitted) Quantifying 3-H thymidine incorporation rates by phylogenetically-defined groups of marine planktonic bacteria. *Appl. Env. Microbiol.*

Van Mooy, B.A.S., Devol, A.H., and Keil, R.G. (in preparation) Planktonic bacterial community structure impacts the cycling of bioavailable dissolved organic carbon. *Limnol. Oceanogr.*

Devol, A.H., Van Mooy, B.A.S., and Keil, R.G. (2002) Thymidine incorporation by specific bacterioplankton in Puget Sound, a quantitative, phylogenetically-based method to separate DNA. American Society of Limnology and Oceanography, Summer Meeting.

Van Mooy, B.A.S., Grocock, J., Devol, A.H., and Keil, R.G. (2002) Bacterioplankton community structure affects response to bioavailable DOC in the eastern subarctic Pacific. American Society of Limnology and Oceanography, Summer Meeting.

Van Mooy, B.A.S., Devol, A.H., and Keil, R.G. (2001) Planktonic bacteria and archaea in a coastal bay. American Society of Limnology and Oceanography, Winter Meeting.